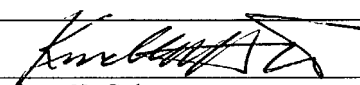


JC07 Rec'd PCT/PTO 28 FEB 2002

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY DOCKET NO. 7388/72613
<b>TRANSMITTAL LETTER TO THE UNITED STATES</b>		
<b>DESIGNATED/ELECTED OFFICE (DO/EO/US)</b> <b>CONCERNING A FILING UNDER 35 USC 371 AND 37 CFR 1.491</b>		U.S. APPLICATION NO. (New Application) <b>10/069723</b>
INTERNATIONAL APPLICATION NO. PCT/JP00/05930	INTERNATIONAL FILING DATE August 31, 2000	PRIORITY DATE CLAIMED August 31, 1999
TITLE OF INVENTION HUMAN 1p36 HOMOZYGOUS DELETION REGION		
APPLICANT(S) FOR DO/EO/US NAKAGAWARA, Akira		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 USC 371(f)). 4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (PCT Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 USC 371(c)(2)) a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 USC 371(c)(2)). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3)) a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 USC 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)). 11. Nucleotide and/or Amino Acid Sequence Submission a. <input type="checkbox"/> Computer Readable Form (CRF) b. Specification Sequence Listing on: i. <input type="checkbox"/> CD-ROM or CD-R (2 copies); or ii. <input checked="" type="checkbox"/> Paper Copy c. <input type="checkbox"/> Statement verifying identity of above copies <b>Items 12 to 19 below concern other document(s) or information included:</b> 12. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. <input type="checkbox"/> Form PTO-1449 <input type="checkbox"/> Copies of Listed Documents 13. <input checked="" type="checkbox"/> An assignment for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 14. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input checked="" type="checkbox"/> Application Data Sheet Under 37 CFR 1.76 18. <input checked="" type="checkbox"/> Return Receipt Postcard 19. <input checked="" type="checkbox"/> Other items or information: Inventor's Data Sheet; PCT/IB/304, PCT/IB/308& International Search Report		

10069723 022002

JC13 Rec'd PCT/PTO 28 FEB 2002

U.S. APPLICATION NO. (New Application) <b>10/069723</b>		INTERNATIONAL APPLICATION NO. PCT/JP00/05930		ATTORNEY DOCKET NO. 7388/72613	
20. ■ The following fees are submitted:				CALCULATION S	PTO USE ONLY
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b>					
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1,040.00					
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$890.00					
International preliminary examination fee (37 CFR 1.482) not paid to USPTO, but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$740.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$710.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1) to (4) ..... \$100.00					
<b>ENTER APPROPRIATE BASIC FEE AMOUNT=</b>				\$890.00	
Surcharge of for furnishing the National fee or oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	14 -20=	0	x \$ 18.00	\$	
Independent Claims	2 - 3 =	0	x \$ 84.00	\$	
■ Multiple Dependent Claim(s) (if applicable)			+\$280.00	\$280.00	
<b>TOTAL OF ABOVE CALCULATIONS=</b>				\$1,170.00	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$	
<b>SUBTOTAL=</b>				\$	
Processing fee of <b>\$130.00</b> for furnishing English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date.				\$	
<b>TOTAL NATIONAL FEE=</b>				\$1,170.00	
Fee for recording the enclosed assignment. The assignment must be accompanied by an appropriate cover sheet. <b>\$40.00</b> per property				\$40.00	
<b>TOTAL FEE ENCLOSED=</b>				\$1,210.00	
				Amount to be: refunded	\$
				charged:	\$
<p>a. <input type="checkbox"/> A check in the amount of \$ to cover the above fee is enclosed.</p> <p>b. ■ Please charge Deposit Account No. 06-1135, under Order No. 7388/72613, in the amount of \$930.00 to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. ■ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1135. A duplicate copy of this sheet is enclosed.</p> <p><b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b></p>					
SEND ALL CORRESPONDENCE TO:			 Kendrew H. Colton Registration No. 30,368 Date: February 28, 2002		
FITCH, EVEN, TABIN & FLANNERY 1801 K Street, N.W., Suite 401L Washington, DC 20005-3960 (202) 419-7007 (telephone) (202) 419-7007 (facsimile)					

**PATENT**  
Attorney Docket No. 7388/72613

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:

NAKAGAWARA, Akira

Application No.: Not Yet Assigned

Filed: Herewith

For: HUMAN 1p36 HOMOZYGOUS DELETION REGION

February 28, 2002

**PRELIMINARY AMENDMENT**

Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Please enter this Preliminary Amendment.

IN THE SPECIFICATION:

Page 1, after the title, insert:

This application is the National phase of International Application PCT/JP00/05930, filed August 31, 2000, which designated the U.S. and that International Application was not published under PCT Article 21(2) in English.

Page 5, beginning at line 10, through page 6, ending at line 17, replace the paragraph with:

Fig. 2 is a photograph of 2% agarose gel electrophoresis of PCR products for NB-1 and MASS-NB-SCH-1 genomic DNA using the D1S2021, PGD, SGC-30343, D1S1768 and D1S2736 primer sets. All of the PCR products include primers that specifically amplify the region of human Neuro Growth Factor  $\beta$  (hNGF $\beta$ ) as positive controls. (Lane 1: empty lane, Lane 2: molecular weight marker (1030, 734, 341, 258, 192, 105, 78, 75, 46, 36, 18, 17, 12, 11, 8 bp from top), Lane 3: positive control (SY5Y), Lane 4: NB-1, Lane 5: MASS-NB-SCH-1, Lane

6: negative control (sterile distilled water), Lane 7: positive control (SY5Y), Lane 8: NB-1, Lane 9: MASS-NB-SCH-1, Lane 10: negative control (sterile distilled water), Lane 11: positive control (SY5Y), Lane 12: NB-1, Lane 13: MASS-NB-SCH-1, Lane 14: negative control (sterile distilled water), Lane 15: positive control (SY5Y), Lane 16: NB-1, Lane 17: MASS-NB-SCH-1, Lane 18: negative control (sterile distilled water), Lane 19: positive control (SY5Y), Lane 20: NB-1, Lane 21: MASS-NB-SCH-1, Lane 22: negative control (sterile distilled water), Lane 23: molecular weight marker (same as Lane 2)).

Page 6, beginning at line 19, through page 7, ending at line 17, replace the paragraph with:

Fig. 6 is a pair of photographs of electrophoresis of PCR products for NB-1 and MASS-NB-SCH-1 genomic DNA using primers designed based on PAC clone data. The gel used was 2.5 agarose. (Lane 1: marker (1202 bp, 517 bp, 396 bp, 201 bp), Lane 2: positive control (human placenta DNA), Lane 3: normal cells derived from neuroblastoma patient without 1p36 deletion, Lane 4: tumor cells derived from neuroblastoma patient without 1p36 deletion, Lane 5: normal cells derived from MASS-NB-SCH-1 patient, Lane 6: MASS-NB-SCH-1, Lane 7: NB1, Lane 8: positive control (mouse hybridoma with human chromosome), Lane 9: positive control (human lymphocytes), Lane 10: positive control (human placenta DNA), Lane 11: normal cells derived from neuroblastoma patient without 1p36 deletion, Lane 12: tumor cells derived from neuroblastoma patient without 1p36 deletion, Lane 13: normal cells derived from MASS-NB-SCH-1 patient, Lane 14: MASS-NB-SCH-1, Lane 15: NB1, Lane 16: positive control (mouse hybridoma with human chromosome), Lane 17: positive control (human lymphocytes), Lane 18: empty lane). In A, dJ1028013-spb was used as primer for Lanes 1-8; dJ142A6-T7 was used as primer for Lanes 9-17. In B, dJ371E1-SP6 was used as primer for Lanes 1-8; dJ587c9-SP6 was used as primer for Lanes 9-17.

Page 5, beginning at line 10, through page 6, ending at line 17, replace the paragraph with:



Identification of the tumor suppressor gene is possible by analysis of Loss of Heterozygosity (hereunder also abbreviated as LOH). The chromosomes of human cells, except for the sex chromosomes, exist as pairs, and two copies of each gene are present, one from the mother and one from the father. According to the "two-hit theory" advocated by Kundson A.G. et al. (Kundson A.G., *Pediatr. Res.*, 10:513- (1976)), inactivation of a gene is only found to occur when both of each copy from the mother and father are inactivated, and LOH is observed most often by this mechanism. LOH refers to a pattern whereby a small genetic abnormality (deletion or substitution of a base or part of the gene) exists in one copy, while the chromosome on which the other copy resides undergoes the entire deletion of a large region including the gene. Consequently, in patients experiencing onset of cancer due to inactivation of a tumor suppressor gene, it is thought that there are two copies of the chromosome on which the tumor suppressor gene resides in normal cells, and one copy in tumor cells.

Page 14, beginning at line 15, replace the paragraph with:

Two of the obtained confluent culturing plates were used to obtain cells by trypsin treatment. The obtained cells were suspended in 5 ml of TEN buffer (TEN: 50 mM Tris-Cl (pH 8.0), 1 mM EDTA, 100 mM NaCl) and homogenized. To the homogenized suspension there were added 750  $\mu$ l of SDS (10%) and 125  $\mu$ l of Proteinase K (MERCK Co., 20 mg/ml), and the mixture was gently mixed by inversion and incubated overnight at 50°C for lysis of the cells.

Page 15, beginning at line 8, replace the paragraph with:

Next, 10.2 ml of ethanol which had been precooled at -20°C was added to and mixed with the collected supernatant, the resulting filamentous DNA was collected with a Pasteur pipette, the excess ethanol was removed and the DNA was dried. An appropriate amount of TE buffer (Tris-Cl (pH 8.0), 1 mM EDTA) was added to the dried DNA, and mixing was effected at room temperature for 1-2 days to dissolve the DNA.

(Example 2) Purification of genomic DNA extracted from human neuroblastoma cell lines.

Page 19, beginning at line 18, replace the paragraph with:

A band near 169 bases amplified by D1S548 as the positive control primer set was observed in all of the cell lines. In contrast, a bands near 285-296 bases amplified by D1S244 were observed only in lines NB-1 and MASS-NB-SCH-1..

(Example 4)

Page 32, beginning at line 3, replace the paragraph with:

In order to obtain the base sequence data for the deletion region, a search was made of an internet gene map database (a database containing recorded human genome data for over 30,000 mapped genes, ESTs etc. according to chromosome: <http://www.ncbi.nlm.nih.gov/genemap99/>) with the keywords "primer D1S244", etc. which were used in the aforementioned examples, resulting in numerous hits for genes and ESTs. Upon conducting a DNA sequence homology search through the internet (NCBI BLAST (National Center of Biotechnology Information USA, <http://www.ncbi.nlm.nih.gov/BLAST>)) for portions of the genome sequence, some ESTs were found. The data for these sequences are shown in Table 7, and those with recorded base sequences are listed in the Sequence Listing.

IN THE CLAIMS:

Please add the following new claims:

6. (New) A homozygous deletion region present in common at position 36 on the short arm of chromosome 1 of human neuroblastoma (1p36).

7. (New) The region according to claim 6, characterized by being deleted in the human neuroblastoma cell lines NB-1 and MASS-NB-SCH-1.

8. (New) The region according to claim 6, characterized by being defined by at least the primer set SGC30343 and D1S2736.

9. (New) The region according to claim 6, characterized by being defined by at least from between a primer set at the SP6 end of the PAC clone dJ1028O13 and a primer set at the SP6 end of the PAC clone dJ371E1 to between the primer set D1S2736 and a primer set at the T7 end of the PAC clone dJ142A6.

10. (New) The region according to claim 9, characterized by being further defined by at least the primer set SGC30343 and D1S2736.

11. (New) The region according to claim 9, characterized by being deleted in the human neuroblastoma cell lines NB-1 and MASS-NB-SCH-1.

12. (New) The region according to claim 8, characterized by being deleted in the human neuroblastoma cell lines NB-1 and MASS-NB-SCH-1.

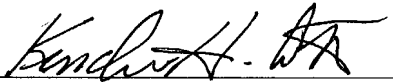
Please see Appendix for amendments.

**REMARKS**

Please enter this Preliminary Amendment. It makes proper PCT cross-reference, (self-evident, clerical amendments to the specification, and presents additional claims. Applicants reserve the right to file a substitute specification with the aforesaid amendments for publication.

Respectfully submitted,

FITCH, EVEN, TABIN & FLANNERY

By: 

Kendrew H. Colton

Registration No. 30,368

Telephone No. (202) 419-7000

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**APPENDIX**  
**AMENDMENTS TO THE SPECIFICATION**

Insertion of paragraph, at page 1, after the title:

This application is the National phase of International Application PCT/JP00/05930, filed August 31, 2000, which designated the U.S. and that International Application was not published under PCT Article 21(2) in English.

Amendments to the paragraph at page Page 5, beginning at line 10, through page 6, ending at line 17:

Fig. 2 is a photograph of 2% agarose gel electrophoresis of PCR products for NB-1 and MASS-NB-SCH-1 genomic DNA using the D1S2021, PGD, SGC-30343, D1S1768 and D1S2736 primer sets. All of the PCR products include primers that specifically amplify the region of human ~~Nerve~~ Neuro Growth Factor  $\beta$  (hNGF $\beta$ ) as positive controls. (Lane 1: empty lane, Lane 2: molecular weight marker (1030, 734, 341, 258, 192, 105, 78, 75, 46, 36, 18, 17, 12, 11, 8 bp from top), Lane 3: positive control (SY5Y), Lane 4: NB-1, Lane 5: MASS-NB-SCH-1, Lane 6: negative control (sterile distilled water), Lane 7: positive control (SY5Y), Lane 8: NB-1, Lane 9: MASS-NB-SCH-1, Lane 10: negative control (sterile distilled water), Lane 11: positive control (SY5Y), Lane 12: NB-1, Lane 13: MASS-NB-SCH-1, Lane 14: negative control (sterile distilled water), Lane 15: positive control (SY5Y), Lane 16: NB-1, Lane 17: MASS-NB-SCH-1, Lane 18: negative control (sterile distilled water), Lane 19: positive control (SY5Y), Lane 20: NB-1, Lane 21: MASS-NB-SCH-1, Lane 22: negative control (sterile distilled water), Lane 23: molecular weight marker (same as Lane 2)).

Amendments to the paragraph at page 6, beginning at line 19, through page 7, ending at line 17:

Fig. 6 is a pair of photographs of electrophoresis of PCR products for NB-1 and MASS-NB-SCH-1 genomic DNA using primers designed based on PAC clone data. The gel used was

2.5 agarose. (Lane 1: marker (1202 bp, 517 bp, 396 bp, 201 bp), Lane 2: positive control (human placenta DNA), Lane 3: normal cells derived from neuroblastoma patient without 1p36 deletion, Lane 4: tumor cells derived from neuroblastoma patient without 1p36 deletion, Lane 5: normal cells derived from MASS-NB-SCH-1 patient, Lane 6: MASS-NB-SCH-1, Lane 7: NB1, Lane 8: positive control (mouse hybridoma with human chromosome), Lane 9: positive control (human lymphocytes), Lane 10: positive control (human placenta DNA), Lane 11: normal cells derived from neuroblastoma patient without 1p36 deletion, Lane 12: tumor cells derived from neuroblastoma patient without 1p36 deletion, Lane 13: normal cells derived from MASS-NB-SCH-1 patient, Lane 14: MASS-NB-SCH-1, Lane 15: NB1, Lane 16: positive control (mouse hybridoma with human chromosome), Lane 17: positive control (human lymphocytes), Lane 18: empty lane). In A, dJ1028013-spb was used as primer for Lanes 1-8; dJ142A6-T7 was used as primer for Lanes 9-17. In B, dJ371E1-SP6 was used as primer for Lanes 1-8; dJ587c9-SP6 was used as primer for Lanes 9-17.

Amendments to the paragraph at page 5, beginning at line 10, through page 6, ending at line 17:

Identification of the tumor suppressor gene is possible by analysis of Loss of Heterozygosity (hereunder also abbreviated as LOH). The chromosomes of human cells, except for the sex chromosomes, exist as pairs, and two copies of each gene are present, one from the mother and one from the father. According to the "two-hit theory" advocated by Kundson A.G. et al. (Kundson A.G., *Pediatr. Res.*, 10:513- (1976)), inactivation of a gene is only found to occur when both of each copy from the mother and father are inactivated, and LOH is observed most often by this mechanism. LOH refers to a pattern whereby a small genetic abnormality (deletion or substitution of a base or part of the gene) exists in one copy, while the chromosome on which the other copy resides undergoes the entire deletion of a large region including the gene. Consequently, in patients experiencing onset of cancer due to inactivation of a tumor suppressor gene, it is thought that there are two copies of the chromosome on which the tumor suppressor gene resides in normal cells, and one copy in tumor cells.

Amendments to the paragraph at page 14, beginning at line 15:

Two of the obtained confluent culturing plates were used to obtain cells by trypsin treatment. The obtained cells were suspended in 5 ml of TEN buffer (TEN: 50 mM Tris-Cl HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl) and homogenized. To the homogenized suspension there were added 750  $\mu$ l of SDS (10%) and 125  $\mu$ l of Proteinase K (MERCK Co., 20 mg/ml), and the mixture was gently mixed by inversion and incubated overnight at 50°C for lysis of the cells.

Amendments to the paragraph at page 15, beginning at line 8:

Next, 10.2 ml of ethanol which had been precooled at -20°C was added to and mixed with the collected supernatant, the resulting filamentous DNA was collected with a Pasteur pipette, the excess ethanol was removed and the DNA was dried. An appropriate amount of TE buffer (Tris-ClHCl (pH 8.0), 1 mM EDTA) was added to the dried DNA, and mixing was effected at room temperature for 1-2 days to dissolve the DNA.

(Example 2) Purification of genomic DNA extracted from human neuroblastoma cell lines.

Amendments to the paragraph at page 19, beginning at line 18:

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(Example 4)

Amendments to the paragraph at page 32, beginning at line 3:

In order to obtain the base sequence data for the deletion region, a search was made of an internet gene map database (a database containing recorded human genome data for over 30,000 mapped genes, ESTs etc. according to chromosome:

<http://www.ncbi.nlm.nih.gov/genemap99/>) with the keywords "primer D1S244", etc. which were used in the aforementioned examples, resulting in numerous hits for genes and ESTs.

Upon conducting a DNA sequence homology search through the internet f(NCBI BLAST

(National Center of Biotechnology Information USA, <http://www.ncbi.nlm.nih.gov/BLAST>))  
for portions of the genome sequence, some ESTs were found. The data for these sequences are shown in Table 7, and those with recorded base sequences are listed in the Sequence Listing.

IN THE CLAIMS:

New claims 6-12 are added.



10/pvt

**DESCRIPTION**HUMAN 1p36 HOMOZYGOUS DELETION REGION**Technical Field**

5 This invention relates to a homozygous deletion region (hereunder also referred to simply as "deletion region") present in common at position 36 on the short arm of chromosome 1 (hereunder also referred to as "1p36") of human neuroblastomas.

**Background Art**

10 Cancer-associated genes linked to oncogenesis are known to include tumor suppressor genes which promote oncogenesis when inactivated, and for example, the tumor suppressor genes RB and WT-1 have already been discovered and identified.

15 The RB gene was so named because it was discovered as the gene responsible for retinoblastoma, an ocular cancer of childhood. In most cases of retinoblastoma, region 14 of the long arm of human chromosome 13 (13q14) is shortened (Yunis, JJ. et al., Am. J. Dis. Child. 132, 161-163(1978)), and it has long been  
20 conjectured that inactivation of a gene on this portion results in predisposition to cancer. However, cloning of the gene was difficult, and RB was first identified as a human tumor suppressor gene in 1986 (Friend, SH. et al., Nature, 323, 643-646(1986)).  
25

Wilms' tumor, which is also called nephroblastoma,

is a fetal malignant tumor originating in the nephric  
blastema tissue in infants. Patients often also  
exhibit abnormalities of the genitourinary organs, but  
epidemiological data tend to suggest the "two-hit  
5 theory" as with retinoblastoma, leading to conjecture  
on the role of a recessive oncogene. In the case of  
patients with WAGR syndrome which also exhibit aniridia  
(A) and mental retardation (R) in addition to the tumor  
(W) and genitourinary (G) abnormalities, a  
10 microdeletion is often found in region 13 on the short  
arm of chromosome 11 (11p13). WT1 has been isolated as  
the gene located in this common deletion region (Call,  
KM. et al., Cell 60, 509-520(1990)).

Numerous tumor suppressor genes have since been  
15 discovered based on the data for chromosome deletion  
regions, but as of the current time no tumor suppressor  
gene for neuroblastoma has been identified from the  
1p36 deletion region.

#### **Disclosure of the Invention**

20 It is an object of this invention to provide data  
relating to a novel homozygous deletion region at  
position 36 on the short arm of chromosome 1 which is  
common to human neuroblastomas.

25 It is another object to provide a method for  
diagnosis and prognosis of tumors based on the base  
sequence data for this region, as well as a tumor

marker which can be used therefor.

It is yet another object to develop a method of gene diagnosis and an anticancer agent, and to develop a method of analyzing the tumor pathology mechanism and a reagent to be used for analysis of the tumor pathology mechanism, based on this base sequence data.

As a result of much diligent research toward finding a tumor suppressor gene for neuroblastoma, the present inventors have discovered a novel homozygous deletion region at position 36 on the short arm of chromosome 1 of NB-1, a human neuroblastoma cell line.

This finding made it possible to design a method for tumor diagnosis and prognosis and a tumor marker which can be used therefor, and this invention was thus completed. The base sequence data for this region also allowed development of a tumor gene diagnosis method and an anticancer agent, as well as development of a method of analyzing the tumor pathology mechanism and a reagent to be used for analysis of the tumor pathology mechanism, and this invention was thereupon further completed.

More specifically, the invention relates to a homozygous deletion region present in common at position 36 on the short arm of chromosome 1 of human neuroblastomas.

Even more specifically, this region is a region

characterized by being defined by at least from between  
a primer set at the SP6 end of the PAC clone dJ1028013  
and a primer set at the SP6 end of the PAC clone  
dJ371E1 to between a primer set D1S2736 and a primer  
5 set at the T7 end of the PAC clone dJ142A6.

The region is also a region characterized by being  
further defined by at least the primer set SGC30343 and  
D1S2736.

The region is yet also a region characterized by  
10 being deleted in the human neuroblastoma cell lines NB-  
1 and MASS-NB-SCH-1.

The invention further includes a tumor marker, a  
tumor diagnostic reagent, a tumor prognostic reagent  
and an anticancer agent which are based on the base  
15 sequence data obtained from this region.

The invention still further includes a tumor  
pathology mechanism analysis method and a tumor  
pathology mechanism analysis reagent which are based on  
the base sequence data obtained from this region.

## 20 **Brief Description of the Drawings**

Fig. 1 is a photograph of 4% agarose gel  
electrophoresis of a PCR product for human  
neuroblastoma genomic DNA using the D1S548 and D1S244  
primer sets. (Lane 1: molecular weight marker (1330,  
25 828, 750, 696, 536, 517, 501, 396, 351, 221, 75, 65, 35,  
22 bp from top), Lane 2: SK-N-BE, Lane 3: SK-N-DZ, Lane

4: RT-BM1, Lane 5: NLF, Lane 6: LA-N-5, Lane 7: IMR32,  
 Lane 8: NMB, Lane 9: OAN, Lane 10: NGP, Lane 11: NB1,  
 Lane 12: CHP134, Lane 13: KCN, Lane 14: NBL-S, Lane 15:  
 CHP901, Lane 16: SY5Y, Lane 17: GANB, Lane 18: SK-N-AS,  
 5 Lane 19: KAN, Lane 20: LHN, Lane 21: SAN, Lane 22: TGW,  
 Lane 23: positive control (human lymphocytes), Lane 24:  
 MASS-NB-SCH-1, Lane 25: negative control (sterile  
 distilled water), Lane 26: molecular weight marker  
 (same as Lane 1)).

10 Fig. 2 is a photograph of 2% agarose gel  
 electrophoresis of PCR products for NB-1 and MASS-NB-  
 SCH-1 genomic DNA using the D1S2021, PGD, SGC-30343,  
 D1S1768 and D1S2736 primer sets. All of the PCR  
 products include primers that specifically amplify the  
 15 region of human Nerve Growth Factor  $\beta$  (hNGF $\beta$ ) as  
 positive controls. (Lane 1: empty lane, Lane 2:  
 molecular weight marker (1030, 734, 341, 258, 192, 105,  
 78, 75, 46, 36, 18, 17, 12, 11, 8 bp from top), Lane 3:  
 positive control (SY5Y), Lane 4: NB-1, Lane 5: MASS-NB-  
 20 SCH-1, Lane 6: negative control (sterile distilled  
 water), Lane 7: positive control (SY5Y), Lane 8: NB-1,  
 Lane 9: MASS-NB-SCH-1, Lane 10: negative control  
 (sterile distilled water), Lane 11: positive control  
 (SY5Y), Lane 12: NB-1, Lane 13: MASS-NB-SCH-1, Lane 14:  
 25 negative control (sterile distilled water), Lane 15:  
 positive control (SY5Y), Lane 16: NB-1, Lane 17: MASS-

NB-SCH-1, Lane 18: negative control (sterile distilled water), Lane 19: positive control (SY5Y), Lane 20: NB-1, Lane 21: MASS-NB-SCH-1, Lane 22: negative control (sterile distilled water), Lane 23: molecular weight marker (same as Lane 2)).

Fig. 3 is a photograph of 5% acrylamide electrophoresis of a PCR product for a PAC clone DNA using the D1S244 primer set. (Lane 1: molecular weight marker (1202 bp, 517 bp, 396 bp, 201 bp), Lanes 2-4: dJ142A6, Lane 5-7: dJ332G17, Lane 8-10: dJ369D13, Lane 11: positive control (human placenta DNA), Lane 12: negative control (sterile distilled water)).

Fig. 4 is a contig map for the PAC18 clone included in lp36 found in strains NB-1 and MASS-NB-SCH-1 by the 1st screening.

Fig. 5 is a contig map for the PAC34 clone included in lp36 found in strains NB-1 and MASS-NB-SCH-1 in the final screening.

Fig. 6 is a pair of photographs of electrophoresis of PCR products for NB-1 and MASS-NB-SCH-1 genomic DNA using primers designed based on PAC clone data. The gel used was 2.5 agarose. (Lane 1: marker (1202 bp, 517 bp, 396 bp, 201 bp), Lane 2: positive control (human placenta DNA), Lane 3: normal cells derived from neuroblastoma patient without lp36 deletion, Lane 4: tumor cells derived from neuroblastoma patient without

1p36 deletion, Lane 5: normal cells derived from MASS-NB-SCH-1 patient, Lane 6: MASS-NB-SCH-1, Lane 7: NB1, Lane 8: positive control (mouse hybridoma with human chromosome), Lane 9: positive control (human lymphocytes), Lane 10: positive control (human placenta DNA), Lane 11: normal cells derived from neuroblastoma patient without 1p36 deletion, Lane 12: tumor cells derived from neuroblastoma patient without 1p36 deletion, Lane 13: normal cells derived from MASS-NB-SCH-1 patient, Lane 14: MASS-NB-SCH-1, Lane 15: NB1, Lane 16: positive control (mouse hybridoma with human chromosome), Lane 17: positive control (human lymphocytes), Lane 18: empty lane). In A, dJ1028013 was used as primer for Lanes 1-8; dJ142A6-T7 was used as primer for Lanes 9-17. In B, dJ371E1-SP6 was used as primer for Lanes 1-8; dJ587c9-SP6 was used as primer for Lanes 9-17.

Fig. 7 is a diagram showing the genome deletion region area for 1p36 elucidated by this invention.

Fig. 8 is a diagram showing the positions for a minimal PAC clone group included in the genome deletion region area for 1p36 elucidated by this invention.

Fig. 9 is a diagram showing the positions for a minimal PAC clone group included in the genome deletion region area for 1p36 elucidated by this invention, which differs somewhat from the combination shown in

Fig. 8.

**Best Mode for Carrying Out the Invention**

(Novel homozygous deletion region for 1p36)

The present inventors focused on the fact that the  
5 human 1p36 deletion has been found in at least 14  
different human tumors (colon cancer, breast cancer,  
brain tumor, etc.), that several tumor suppressor genes  
are predicted to be present in this region and that the  
deletion has a high correlation with poor prognosis of  
10 neuroblastoma (Schwab M. et al., Genes Chromosomes  
Cancer 16, 211-229(1996), Fong C.T. et al., Cancer Res.  
52, 1780-1785(1992)), and also focused on the fact that  
normalization of cancer cells has been achieved by  
introduction of normal 1p36 in colon cancer (Tanaka K.  
15 et al., Oncogene, 8, 2253-2258(1993)) and that the 1p36  
deletion is found from the initial stages of liver  
cancer and colon cancer (Kuroki T. et al., Genes  
Chromosomes Cancer 13, 163-167(1995)), which suggested  
a high possibility that the 1p36 deletion plays a role  
20 in the early stages of canceration of normal cells.

Identification of the tumor suppressor gene is  
possible by analysis of Loss of Heterozygosity  
(hereunder also abbreviated as LOH). The chromosomes  
of human cells, except for the sex chromosomes, exist  
25 as pairs, and two copies of each gene are present, one  
from the mother and one from the father. According to



the "two-hit theory" advocated by Kundson A.G. et al.  
(Kundson A.G., Pediatr. Res. 513- (1976)), inactivation  
of a gene is only found to occur when both of each copy  
from the mother and father are inactivated, and LOH is  
5 observed most often by this mechanism. LOH refers to a  
pattern whereby a small genetic abnormality (deletion  
or substitution of a base or part of the gene) exists  
in one copy, while the chromosome on which the other  
copy resides undergoes the entire deletion of a large  
10 region including the gene. Consequently, in patients  
experiencing onset of cancer due to inactivation of a  
tumor suppressor gene, it is thought that there are two  
copies of the chromosome on which the tumor suppressor  
gene resides in normal cells, and one copy in tumor  
15 cells.

However, the commonly observed range for LOH is  
very large in terms of the molecular genetic distance,  
and the numbers of genes and numbers of bases contained  
therein are exceedingly huge. The current actual  
20 deletion region of 1p36 is limited to the approximately  
8-10 Mb region of 1p36.2-1p36.3 according to LOH  
analysis, but this region must be further narrowed down.

In order to identify the tumor suppressor gene,  
the present inventors analyzed neuroblastoma cell lines  
25 and discovered an approximately 700 kb homozygous  
deletion region on the neuroblastoma chromosome 1p36.

These 700 kb constituted part of the deletion region (approximately 8-10 Mb) elucidated by analysis of loss of heterozygosity.

(Application example for human neuroblastoma 1p36 homozygous deletion region)

(1) Tumor gene diagnosis, method of prognosis, and tumor marker to be used therefor

The base sequence disclosed by this invention may be used to investigate the presence or absence of the 1p36 homozygous deletion region in cell specimens to allow diagnosis and prognosis of tumors. For investigation of the presence or absence of the deletion region there may be applied all of the methods using any desired sequence of the 1p36 homozygous deletion region and base sequences (probes) that can hybridize thereto. When necessary, these probes may be labeled with radioisotopes, enzymes (for example, alkali phosphatase or peroxidase), fluorescent compounds (for example, fluorescein) or chemiluminescent compounds (for example, acridinium esters or isoluminol). That is, when nucleic acids that hybridize to the probe sequences are not present in the cell specimen, a high possibility exists for tumor onset, while in specimens from known cancer onset cases the prognosis can be predicted to be poor. As hybridization methods there may be used, for example,

Southern blotting, Northern blotting, dot blotting, Fluorescence *in situ* Hybridization (FISH) or the like.

For investigation of the presence or absence of the deletion region there may also be used a Polymerase Chain Reaction (PCR) with any desired sequences from the 1p36 homozygous deletion region as the primers.

Identification of loss of heterozygosity may be accomplished using, for example, Restriction Fragment Length Polymorphism (RFLP).

These methods are carried out according to protocols known by skilled professionals. Reference may be made to, for example, Molecular Cloning, A Laboratory Manual (T. Maniatis: Cold Spring Harbor Laboratory Press) and Idenshibyo Nyumon [Introduction to Genetic Diseases] (F. Takahisa: Nankodo Publishing).

## (2) Development of anticancer agent

Cells having the 1p36 homozygous deletion or loss of heterozygosity can be found using base sequences disclosed by the invention, thus allowing screening of anticancer agents. Because the cells are cancerous, their cell division occurs faster than that of normal cells. By adding candidate anticancer agents to the cell culture solution and observing the rate of cell division, it is possible to screen for anticancer agents against tumors having the 1p36 homozygous deletion or loss of heterozygosity. The cell division

rate can be measured based on, for example, the amount of uptake of radioisotope (for example, tritium)-labeled thymidine.

(3) Method of analyzing tumor pathology mechanism

5        Cells having the 1p36 homozygous deletion or loss of heterozygosity can be found using base sequences disclosed by the invention, thus allowing analysis of the tumor pathology mechanism. The proteins may be extracted from these and normal cells, and the protein  
10        expressed only by the normal cells identified by, for example, Western blotting. This protein may then be used for identification of other proteins that specifically interact therewith by, for example, affinity chromatography. By repeating such methods it  
15        is possible to identify proteins connected with oncogenesis and thus analyze the tumor pathology mechanism through analysis of their intracellular function.

20        The 1p36 homozygous deletion region common to neuroblastoma which was discovered by this invention will now be described in detail by way of examples.

Examples

(Example 1) Extraction of genomic DNA from human neuroblastomas

25        Each of the following human neuroblastoma-derived cell lines was cultured on a 10 cm culturing plate to

general confluency. The names and sources of the cell lines used are summarized below.

SK-N-BE (ATCC Number: CRL-2271)

SK-N-DZ (ATCC Number: CRL-2149)

5 RT-BM1

NLF: donated by Garrett M. Brodeur

LA-N-5: (Riken Cell Bank (The Institute of Physical and Chemical Research): RC0485)

IMR32: donated by Garrett M. Brodeur

10 NMB: donated by Garrett M. Brodeur

OAN: donated by Garrett M. Brodeur

NGP: donated by Garrett M. Brodeur

NB1: donated by Dr. Takafumi Matsumura of Kyoto

15 Prefectural University, Dept. of Pediatrics (Considered one of the oldest neuroblastoma cell lines in Japan, established at the Niigata University Brain Research Laboratory)

CHP134: donated by Garrett M. Brodeur

KCN: donated by Garrett M. Brodeur

20 NBL-S: donated by Garrett M. Brodeur

CHP901: cell line established from patient surgically extracted specimen

SY5Y: donated by Garrett M. Brodeur

25 GANB: cell line established from patient surgically extracted specimen

SK-N-AS (ATCC Number: CRL-2137)

KAN: donated by Garrett M. Brodeur

LHN: donated by Garrett M. Brodeur

SAN: donated by Garrett M. Brodeur

TGW: donated by Garrett M. Brodeur

5 MASS-NB-SCH-1: donated by Dr. Hisayuki Hiraiwa of  
Shizuoka Children's Hospital, Clinical Pathology  
Section. Cancer 79, 2036-2044 (1997)

KP-N-NB: donated by Dr. Takafumi Matsumura of Kyoto  
Prefectural University, Dept. of Pediatrics

10 NB69: (Riken Cell Bank: RC0480)

NBTu-1: donated by Dr. Kinji Yokomori of Japanese Red  
Cross Medical Center, Dept. of Pediatrics

SK-N-MC (ATCC Number: HTB-10)

SK-N-SH (ATCC Number: HTB-11).

15 Two of the obtained confluent culturing plates  
were used to obtain cells by trypsin treatment. The  
obtained cells were suspended in 5 ml of TEN buffer  
(TEN: 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl)  
and homogenized. To the homogenized suspension there  
20 were added 750 µl of SDS (10%) and 125 µl of Proteinase  
K (MERCK Co., 20 mg/ml), and the mixture was gently  
mixed by inversion and incubated overnight at 50°C for  
lysis of the cells.

The next day, 5.1 ml of saturated phenol was added,  
25 and then the mixture was mixed for one hour and  
centrifuged at 10,000 rpm for 10 minutes, after which

the supernatant was collected. This was further centrifuged at 3,000 rpm for 10 minutes and the supernatant was collected.

5 After adding 5.1 ml of chloroform:isoamyl alcohol = 24:1 to the collected supernatant and mixing for one hour, the mixture was centrifuged at 3000 rpm for 10 minutes and the supernatant was collected.

10 Next, 10.2 ml of ethanol which had been precooled at -20°C was added to and mixed with the collected supernatant, the resulting filamentous DNA was collected with a Pasteur pipette, the excess ethanol was removed and the DNA was dried. An appropriate amount of TE buffer (Tris-HCl, (pH 8.0), 1 mM EDTA) was added to the dried DNA, and mixing was effected at room temperature for 1-2 days to dissolve the DNA.

15 (Example 2) Purification of genomic DNA extracted from human neuroblastoma cell lines

20 The genomic DNA extracted from human neuroblastoma cell lines was purified under the following conditions using a QIAamp Blood Kit (QIAGEN).

The extracted genomic DNA was quantified by ultraviolet absorption at 260 nm, and to 5-10 µg of the DNA there was added an appropriate amount of TE buffer to 225 µl. After further adding 210 µl of ethanol, the mixture was stirred.

The stirred solution was transferred to a QIAamp

column (kit accessory, QIAGEN), and centrifugation was performed at 8,000 rpm for one minute to adsorb the genomic DNA onto the column. A 500 µl portion of Buffer AW (kit accessory, QIAGEN) was added to the  
5 column, and centrifugation was performed at 8,000 rpm for one minute. After then further adding Buffer AW and centrifuging at 15,000 rpm for 3 minutes, the column was transferred to a 1.5 ml fresh tube, 200 µl of Buffer AE prewarmed to 70°C was added, and the  
10 column was allowed to stand at room temperature for one minute and then centrifuged at 8,000 rpm for one minute to purify the DNA.

The purified genomic DNA was quantified based on the ultraviolet absorption at 260 nm.

15 (Example 3) Identification of deletion lines by 1p36 site-specific PCR using human neuroblastoma genomic DNA

The genomic DNA of the 27 human neuroblastoma cell lines extracted and purified in Examples 1 and 2 was used for identification of the 1p36 deletion by the  
20 following site-specific PCR. The PCR primers used were products of Sawady Technology Co., Ltd., unless otherwise specified.

A 10 µl mixture was prepared by combining 1 µl of genomic DNA solution (2 ng/µl), 1 µl of 10xPfu buffer,  
25 1 µl of dNTPs (2 mM), 0.2 µl of D1S548F (20 pmole/µl), 0.2 µl of D1S548R (20 pmole/µl), 0.2 µl of D1S244F (20



pmole/ $\mu$ l), 0.2  $\mu$ l of D1S244R (20 pmole/ $\mu$ l), 6.12  $\mu$ l of sterile distilled water and 0.08  $\mu$ l of Pfu.

The D1S548 and D1S244 are both known primer sets capable of specifically amplifying the 1p36.23-31 region, and their sequences are shown in Table 1.

Table 1 Sequences of D1S548 and D1S244

Primer set	Sequence(F) 5'-3' (SEQ. ID. No.)	Sequence(R) 5'-3' (SEQ. ID. No.)	Position on chromosome	Amplified size
D1S548	GAACTCATTTGGCAAAGGAA (25)	GCCTCTTTGTTGCAGTGATT (28)	1p36.23-31	near 167 bp
D1S244	GAGCAGCACCGTACAAAT (26)	AGCTCCGCTCCCTGTAAT (7)	1p36.23-31	near 285-296 bp

The 1.3 Mb chromosome telomere end was amplified by D1S548 and D1S244. F and R indicate the directions of the primers.

When absolutely no deletion is present in the  
5 lp36.23-31 region, an amplified band is observed near  
167 bases for D1S548 and near 285-296 bases for D1S244.  
When a deletion is present in the lp36.23-31 region  
specified by this invention, a band is observed near  
167 bases for D1S548, but no amplified band is observed  
10 near 285-296 bases for D1S244.

After denaturing the mixture at 96°C for 3 minutes,  
it was provided for PCR with 30 repeated cycles of 94°C  
for 30 seconds, 56°C for 45 seconds and 72°C for one  
minute, followed by a final incubation at 72°C for 10  
15 minutes. The obtained PCR product was subjected to  
electrophoresis using 4% agarose gel. The results are  
shown in Fig. 1.

A band near 169 bases amplified by D1S548 as the  
positive control primer set was observed in all of the  
20 cell lines. In contrast, a band near 285-296 bases  
amplified by D1S244 was observed only in lines NB-1 and  
MASS-NB-SCH-1.

(Example 4)

A PCR was conducted with each of 5 other primer  
25 sets, SGC-30343, D1S2021, D1S1768, PGD and D1S2736,  
which are known to specifically amplify the lp36.23-31

region of the two cell lines NB-1 and MASS-NB-SCH-1.  
 As a positive control, there were used two different  
 types of human Nerve Growth Factor  $\beta$  (hNGF $\beta$ ) located  
 at position 13 of the short arm of human chromosome 1  
 5 (1p13). hNGF $\beta$  60s is a primer with an annealing  
 temperature near 60°C while hNGF $\beta$  57s is a primer with  
 an annealing temperature near 57°C, and the bands are  
 amplified near 253 bp and 229 bp, respectively. The  
 sequences for these primers are shown in Table 2.

Sequence (T)	Sequence (F)
SHGC-31453, SGC-30343, D1S2021, D1S1768, PGD, D1S2736	

Primer set	Sequence (F) 5'-3' (SEQ. ID. No.)	Sequence (R) 5'-3' (SEQ. ID. No.)	Amplified size
SHGC-31453	AAACCTCAGCAGTGGTCCTG (2)	AAACCCCTCTTCTTAGAGTGACG (1)	near 129 bp
SGC-30343	GTGTTGGAGGCCAAATGTG (32)	TTGACCGTGTGTCACCTTAA (39)	near 131 bp
D1S2021	GTGAAGAGCCAGAGATGTCC (30)	CATAGTGCCCAACACGCCAAAG (15)	near 190 bp
D1S1768	ATCTGATGTTTGAGCCTTGAG (13)	ACTAACAGCTCCGTGACGC (5)	near 147 bp
PGD	TTCCAGTTGATCAGAGTGCG (38)	ACCAGGAGCTGCTCATGTG (4)	near 131 bp
D1S2736	CCTCCAGGGTATTCTTGG (18)	TTTTTGAGGTGTGAGAGCAG (42)	near 130 bp

As seen by the results (Fig. 2), there was detected a band for amplification with the hNGF $\beta$  positive controls, but no band was observed for amplification in the 1p36.23-31 region.

5       Based on these results it was determined that the human neuroblastoma lines NB-1 and MASS-NB-SCH-1 have deletions at least in the region from SGC-30343 to D1S2736 of 1p36, on both chromosomes.

(Example 5)   Selection of PAC clones containing  
10   sequence of deletion region

The primer set D1S244 which was used to confirm the 1p36.23-31 region deletion in Example 3 was used for PCR with PAC clones (P1-derived artificial chromosomes, product of Oligo Service, Tsukuba  
15   Laboratory) having the human genome at random, using an LA PCR kit (Takara Shuzo), and PAC clones containing the deletion region sequence were selected. A 20  $\mu$ l mixture was prepared by combining 5  $\mu$ l of PAC DNA pool solution (Z1-Z4: Oligo Service, Tsukuba Laboratory), 2  
20    $\mu$ l of 10xLA PCR buffer, 2.5  $\mu$ l of dNTPs (2 mM), 0.4  $\mu$ l of D1S244F (100 pmole/ $\mu$ l), 0.4  $\mu$ l of D1S244R (100 pmole/ $\mu$ l), 9.6  $\mu$ l of sterile distilled water and 0.1  $\mu$ l of LA Taq (5 u/ $\mu$ l).

After denaturing the mixture at 94°C for 3 minutes,  
25   it was provided for PCR reaction with 35 repeated cycles of 94°C for 30 seconds, 56°C for 30 seconds and

72°C for one minute, followed by a final static incubation at 72°C for 5 minutes.

The PCR product was subjected to electrophoresis, and then only the PAC DNA pools for which an  
5 amplification band near 285-296 bases was observed were subjected to a second PCR by the same procedure to narrow down the clones.

The results are shown in Fig. 3. An amplified  
band near 285-296 bases was observed for the 4 PAC  
10 clones dJ142A6, dJ332G17, dJ345P21 and dJ369D13. Fig. 3 shows the results for only dJ142A6, dJ332G17 and dJ369D13.

As a result of screening in the same manner with the other primer sets used to confirm the 1p36.23-31  
15 region deletion, SHGC-31453, SGC-30343, D1S2021, D1S1768, PGD and D1S2736, there were isolated 14 clones for which amplification was observed with the five primer sets excluding SHGC-31453.

The clones obtained in this manner were taken as  
20 the 1st screening. The 18 clones obtained by the 1st screening are shown in Table 3.

18 PAC clones obtained by 1st screening and their contigs

(Those in bold are clones detected by more than one primer)

24



The total of 18 clones obtained by the 1st screening are believed to retain the genome sequence of the deleted region of the two strains NB-1 and MASS-NB-SCH-1, and since some sequences were detected by multiple primer sets, 3 general contigs were formed (respectively designated as Contig 1, Contig 2 and Contig 3). The contigs for the 18 PAC clones obtained by the 1st screening are shown in Fig. 4.

RPN2434 (Kit: Amersham Co.) was used according to the procedure described below to determine the base sequences at both ends of a total of 9 PAC clones, namely the 2 clones dJ99E23 and dJ371E1 from Contig 1, the 4 clones dJ326J10, dJ739D18, dJ756A17 and dJ373D24 from Contig 2 and the 3 clones dJ369D13, dJ142A6 and dJ345P21 from Contig 3. Sterile distilled water was added to a mixture of 2 µg of PAC DNA, 6 pmol of SP6 or T7 primer and 2 µl of each reagent for A, C, G and T (kit accessories), to prepare a total of 6 µl of the mixture. After denaturing the mixture at 95°C for 2 minutes, it was provided for PCR reaction with 30 repeated cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for one minute. After adding 5 µl of reaction suspending solution (kit accessory), the reaction solution was denatured at 95°C for 2 minutes, cooled on ice, subjected to 6% acrylamide gel electrophoresis and sequenced. Ten new walking primer

sets were designed from the obtained base sequence data.

Table 4 shows the selected clones and the sequences of their respective primer sets.

Table 4

Sequences of primer sets used for 2nd and 3rd screenings

2nd

Contig	PAC clone	Primer set	Sequence(F) 5'-3' (SEQ. ID. No.)	Sequence(R) 5'-3' (SEQ. ID. No.)
1	dJ99E23	dJ99E23-T7	GCGTTAGGAATTGAACAG (29)	TGGGAGTATGCTGCCGG (37)
	dJ371E1	dJ371E1-SP6	CTGCTTCGGAGGTCAGCC (21)	GTGACTGCGTGATGCAT (31)
2	dJ326J10	dJ326J10-SP6	ACTTGGAGGATTTATTG (6)	ATACATAAGATAAGAATC (11)
	dJ739D18	dJ739D18-SP6	TAGCTGAAGCAATCTATT (34)	TTTCCGCTCTTCCACAC (41)
	dJ756A17	dJ756A17-SP6	AGTATGCTGTGTGCCAG (9)	GAGGACTAGTGTCAGAAA (27)
	dJ373D24	dJ373D24-SP6	AGTAGACACGGGTATCAG (8)	CCACCTCCAAATCCTGAC (16)
3	dJ369D13	dJ369D13-T7	CGGCCTTAAATCTGATA (19)	CTCAATACTGGATCTGTG (20)
	dJ142A6	dJ142A6-SP6	AGTATGGCAGACTTGATG (10)	ATGAATGGGACAAATTC (14)
	dJ345P21	dJ345P21-T7	CTGGGCAAGCTAATTTTG (23)	TCTGGACGTACTCTGTTC (35)
			CCCTACCCACACTTCAC (17)	TTTCCTGCGTGTGGCCTC (40)

3rd

Contig	PAC clone	Primer set	Sequence(F) 5'-3' (SEQ. ID. No.)	Sequence(R) 5'-3' (SEQ. ID. No.)
1	dJ587C9	dJ587C9-T7	ATATTGCAGACTGAACAG (12)	AAGCAGTTTTCCCCATAA (3)

The newly designed primer sets were used for  
 reselection of PAC clones from PAC DNA pools by PCR,  
 and the process of designing of walking primers based  
 on both end sequences of the selected clones and  
 5 determination of contigs based on walking was repeated.  
 These were taken as the 2nd and 3rd screenings. Table  
 5 shows the selected clones and the sequences of their  
 respective primers sets.

PAC clones obtained by 2nd and 3rd screenings

(Those in bold are clones detected by more than one primer)

## 3rd

Primer set	Newly obtained PAC clones	Contig
dJ587C9-T7	dJ1028O13, dJ1028N11	1

The sequences of the walking primers used for the 2nd and 3rd screenings are shown in Table 4. Finally, one contig of 34 PAC clones was obtained. The results are shown in Fig. 5.

5 (Example 6) Identification of genome deletion sites on 1p36 of NB-1 and MASS-NB-SCH-1

Based on Example 4 it was judged that NB-1 and MASS-NB-SCH-1 have deletions on both chromosomes in at least the regions defined from SGC-30343 to D1S2736 of 10 1p36, but the following procedure was carried out next to identify both ends of the deletion regions.

New primers were synthesized near the deletion region based on the contig data for the PAC clones obtained in Example 5 and the NB-1 and MASS-NB-SCH-1 15 strains and positive controls were subjected to PCR under the conditions described below, after which the presence or absence of specific band amplification was investigated. An LA PCR kit (Takara Shuzo) was used for PCR in the following manner.

20 A 10  $\mu$ l mixture was prepared by combining 1  $\mu$ l of genomic DNA solution (100 ng/ $\mu$ l), 1  $\mu$ l of 10xLA PCR buffer, 0.6  $\mu$ l of dNTPs (2.5 mM, kit accessories), 0.1  $\mu$ l of Primer F (100 mM), 0.1  $\mu$ l of Primer R (100 mM), 7.15  $\mu$ l of sterile distilled water and 0.05  $\mu$ l of LA 25 Taq (5 u/ $\mu$ l, kit accessory). After denaturing the mixture at 95°C for 2 minutes, it was provided for PCR

5

10

15

20

SP6, dJ142A6-T7

31

(Example 7) Base sequence data for 1p36 deletion region

In order to obtain the base sequence data for the deletion region, a search was made of an internet gene map database (a database containing recorded human  
5 genome data for over 30,000 mapped genes, ESTs etc. according to chromosome:  
<http://www.ncbi.nlm.nih.gov/genemap99/>) with the keywords "primer D1S244", etc. which were used in the  
10 aforementioned examples, resulting in numerous hits for genes and ESTs. Upon conducting a DNA sequence homology search through the internet [NCBI BLAST (National Center of Biotechnology Information USA,  
<http://www.ncbi.nlm.nih.gov/BLAST>)] for portions of the  
15 genome sequence, some ESTs were found. The data for these sequences are shown in Table 7, and those with recorded base sequences are listed in the Sequence Listing.



Table 7 Base sequence data for deletion region

Base sequence	Name or accession No.	Definition or feature	Sequence length (bp)	SEQ. ID. No.
1	UFD2(AF043117)	ubiquitin-fusion degradation protein 2	3909	44
2	KIF1B.	kinesin-like protein KIF1B		
3	CORT(NM001302)	preprocortistatin	318	45
4	PEX-14(AB017546)	peroxisome membrane protein		46
5	KIAA0684(AB014584)	Homo sapiens clone 686 protein (KIAA0684) mRNA.	5312	47
6	KIAA0591(AB011163)	Homo sapiens mRNA for KIAA0591 protein, partial cds.	5368	48
7	AI080353(EST)	ox80f04.s1 Soares NhMPu S1 Homo sapiens cDNA clone	422	49
8	D79099(EST)	HUM531D06B Human placenta polyA+ (T.Fujiwara) Homo sapiens cDNA clone	320	50
9	AA059029(EST)	zf63h02.s1 Soares retina N2b4HR Homo sapiens cDNA clone	446	51
10	AA628589(EST)	af39g05.s1 Soares_total_fetus_Nb2HF8_9w Homo sapiens cDNA clone	443	52
11	AA728950(EST)	nw02c08.s1 NCI CGAP Pr16 Homo sapiens cDNA clone	437	53
12	AA328945(EST)	EST32514 Embryo, 12 week I Homo sapiens cDNA 5' end, mRNA sequence	302	54
13	R89689(EST)	ym99c01.r1 Soares adult brain N2b4HB55Y Homo sapiens cDNA clone	450	55
14	AA018367(EST)	ze41e05.r1 Soares retina N2b4HR Homo sapiens cDNA clone	504	56
15	Z19845(EST)	HSAAAAUKT H, Human adult Brain Cortex tissue Homo sapiens cDNA	281	57
16	AI050021(EST)	an22d04.x1 Gessler Wilms tumor Homo sapiens cDNA clone	434	58
17	R67760(EST)	yi28f03.r1 Soares placenta Nb2HP Homo sapiens cDNA clone	496	59

(Example 8) Sequencing of 1p36 deletion region

A PAC clone group was selected which included essentially all of the deletion region, in the same manner as Example 6. These PAC clones were dJ371E1, dJ756A17, dJ266I16 and dJ345P21, the overall structural map of which is shown in Fig. 9. Thus, by sequencing each of these PAC clones and aligning their sequences, it is possible to determine approximately the entire length base sequence of the deletion region.

The sequences of each of the clones were determined by the nested deletion (ND) method. See Hattori, M., Tanpakushitsu, Kakusan, Kouso [Proteins, Nucleic acids, Enzymes] 42, 17, 296-303(1997) or Hattori, M. et al., Nucl. Acids Res. 25, 1802-1808(1997).

In this manner, the base sequences of each of the clones, including the deletion region, were determined. Specifically, the base sequences of 5 contiguous partial sequences including the deletion region were found for PAC clone dJ371E1. Their partial sequences were designated as dJ371E1-1 (SEQ. ID. No.60), dJ371E1-2 (SEQ. ID. No.61), dJ371E1-3 (SEQ. ID. No.62), dJ371E1-4 (SEQ. ID. No.63) and dJ371E1-5 (SEQ. ID. No.64). The respective base sequences are listed in the Sequence Listing. The base sequences of 7 contiguous partial sequences including the deletion

region were found for PAC clone dJ756A17. Their partial sequences were designated as dJ756A17-1 (SEQ. ID. No.65), dJ756A17-2 (SEQ. ID. No.66), dJ756A17-3 (SEQ. ID. No.67), dJ756A17-4 (SEQ. ID. No.68),  
5 dJ756A17-5 (SEQ. ID. No.69), dJ756A17-6 (SEQ. ID. No.70) and dJ756A17-7 (SEQ. ID. No.71). The respective base sequences are listed in the Sequence Listing. The base sequences of 9 contiguous partial sequences including the deletion region were found for PAC clone  
10 dJ266I16. Their partial sequences were designated as dJ266I16-1 (SEQ. ID. No.72), dJ266I16-2 (SEQ. ID. No.73), dJ266I16-3 (SEQ. ID. No.74), dJ266I16-4 (SEQ. ID. No.75), dJ266I16-5 (SEQ. ID. No.76), dJ266I16-6 (SEQ. ID. No.77), dJ266I16-7 (SEQ. ID. No.78),  
15 dJ266I16-8 (SEQ. ID. No.79) and dJ266I16-9 (SEQ. ID. No.80). The respective base sequences are listed in the Sequence Listing. The base sequences of 8 contiguous partial sequences including the deletion region were found for PAC clone dJ345p-21. Their  
20 partial sequences were designated as dJ345p-21-1 (SEQ. ID. No.81), dJ345p-21-2 (SEQ. ID. No.82), dJ345p-21-3 (SEQ. ID. No.83), dJ345p-21-4 (SEQ. ID. No.84), dJ345p-21-5 (SEQ. ID. No.85), dJ345p-21-6 (SEQ. ID. No.86), dJ345p-21-7 (SEQ. ID. No.87) and dJ345p-21-8 (SEQ. ID. No.88).  
25 The respective base sequences are listed in the Sequence Listing.

(Example 9) Diagnosis for prognosis of human neuroblastoma based on base sequences included in deletion region

5 Cells of patients with pediatric neuroblastoma were used to examine the correlation between the presence or absence of LOH for 1p36 demonstrated here and various factors which are associated with tumor prognosis. The results are shown in Table 8.

Table 8

Prognosis of human neuroblastoma based on LOH for 1p36

(a) Correlation with patient age:  $p=0.008$ 

	With LOH	Without LOH	Total
Age < 1	23	103	126
Age $\geq$ 1	27	51	78
Total	50	154	204

(b) Correlation with origin of onset:  $p=0.000$ 

	With LOH	Without LOH	Total
Adrenal body	42	81	123
Other than adrenal body	8	78	85
Total	50	159	209

5

(c) Correlation with trkA expression level:

 $p=0.003$ 

	With LOH	Without LOH	Total
High trkA expression	24	36	60
Low trkA expression	18	79	97
Total	42	115	157

(d) Correlation with presence of N-myc:  $p=0.000$ 

	With LOH	Without LOH	Total
With N-myc	17	3	180
Without N-myc	31	149	20
Total	48	152	200

10

Generally speaking, patients with high expression of the oncogene trkA are considered to have poor prognosis. See Nakagawara, A. et al., Cancer Res. 52, 1364-1368 (1992), Nakagawara, A. et al., N. Engl. J. Med. 328, 847-854 (1993).

In fact, upon examining LOH for 1p36 across 157 pediatric neuroblastoma patients based on comparison of the degree of trk gene expression, among 42 patients who had LOH on 1p36, 24 patients had high trk expression while 18 had low trk expression. Conversely, among the 115 patients with no LOH on 1p36, 36 patients had high trk expression while 79 had low trk expression. A good correlation was therefore obtained between LOH on 1p36 and trk expression.

Prognosis is generally also poor for patients with high age of onset (Nakagawara A. et al., Med. Pediatr. Oncol. 31, 113-115(1998)), patients with primary lesion in the adrenal body (Nakagawara A. et al., Surgery 104, 34-40(1988)), and patients with high expression of the oncogene N-myc (Brodeur GM. et al., Science 224, 1121-1124(1984)), and a very stable correlation was found with the presence or absence of LOH for 1p36.

This confirms that the presence or absence of LOH for 1p36 can serve as an excellent marker for prognosis and identification.

#### **Industrial Applicability**

According to this invention, there has been identified a homozygous deletion region present in common at position 36 on the short arm of chromosome 1 of human neuroblastoma. Based on the base sequence data of the deletion region it is possible to design a

method for diagnosis, prognosis of tumors, as well as a  
tumor marker which can be used therefor, while the base  
sequence data of the deletion region can also be used  
to develop a method of tumor gene diagnosis and an  
5 anticancer agent, and to develop a method of analyzing  
the tumor pathology mechanism and a reagent to be used  
for analysis of the tumor pathology mechanism.

# CLAIMS

1. A homozygous deletion region present in common at position 36 on the short arm of chromosome 1 of human neuroblastoma (1p36).

2. The region according to claim 1, characterized by being defined by at least from between a primer set at the SP6 end of the PAC clone dJ1028013 and a primer set at the SP6 end of the PAC clone dJ371E1 to between the primer set D1S2736 and a primer set at the T7 end of the PAC clone dJ142A6.

3. The region according to claim 1 or 2, characterized by being further defined by at least the primer set SGC30343 and D1S2736.

4. The region according to claim 1 or 2, characterized by being deleted in the human neuroblastoma cell lines NB-1 and MASS-NB-SCH-1.

5. The region according to claim 3, characterized by being deleted in the human neuroblastoma cell lines NB-1 and MASS-NB-SCH-1.



**ABSRTACT**

There are disclosed base sequence data for a deletion region at position 36 of the short arm of chromosome 1 of the human neuroblastoma cell lines NB-1  
5 and MASS-NB-SCH-1, as a tumor suppressor gene for human neuroblastoma.

Fig. 1



Fig. 2

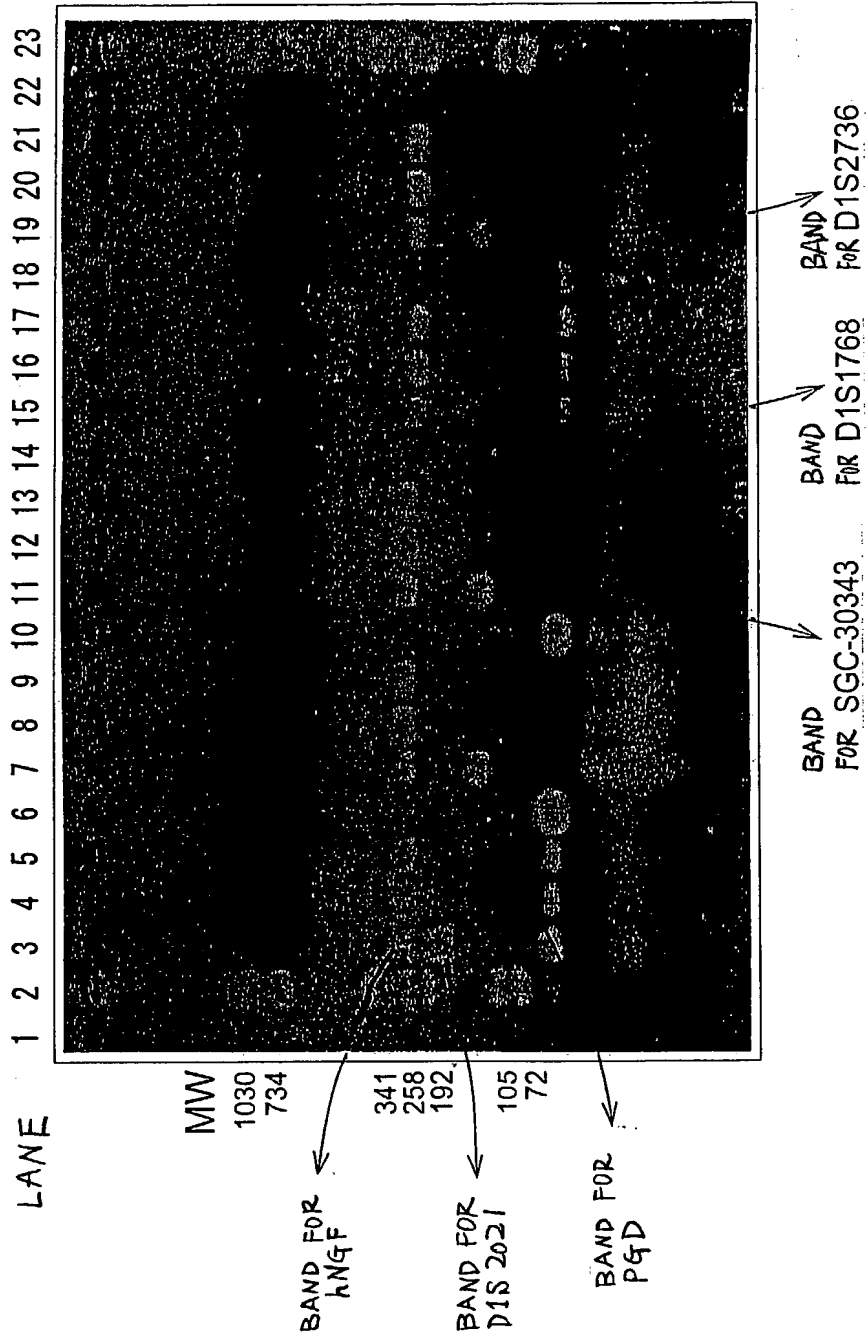
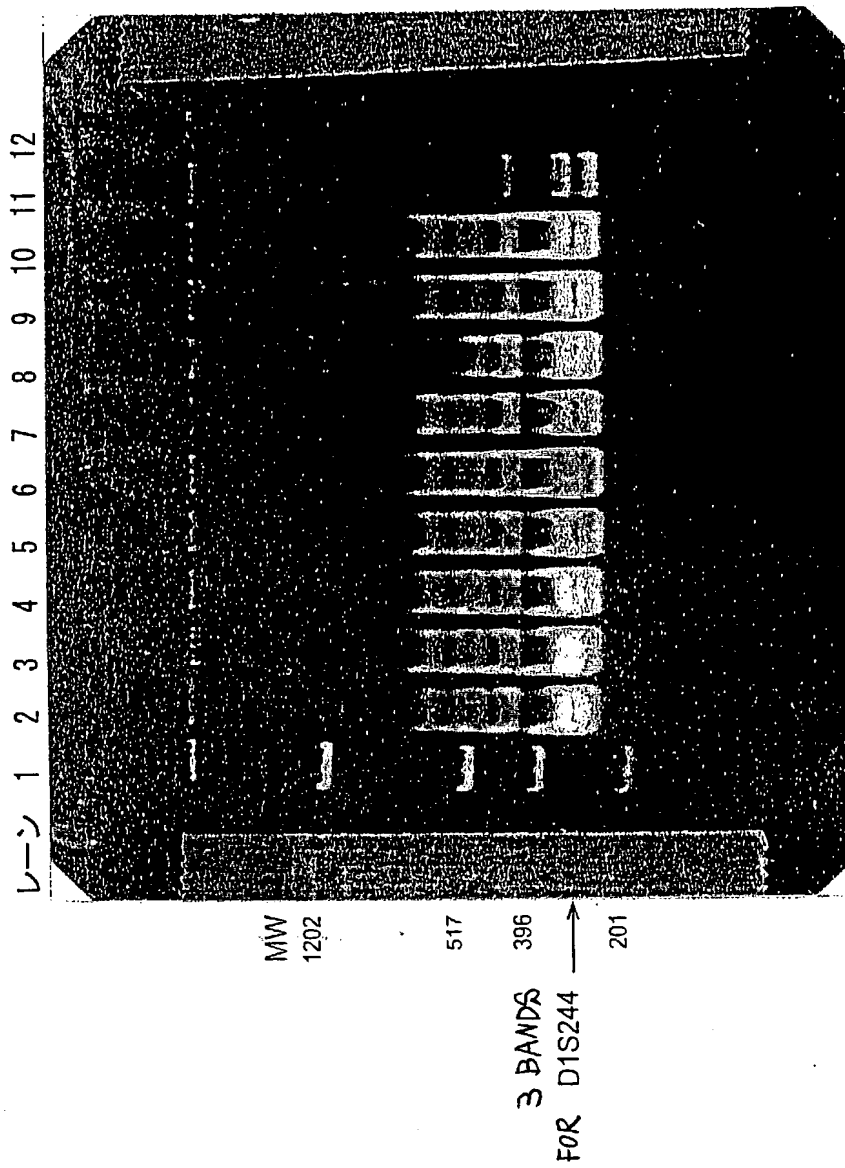
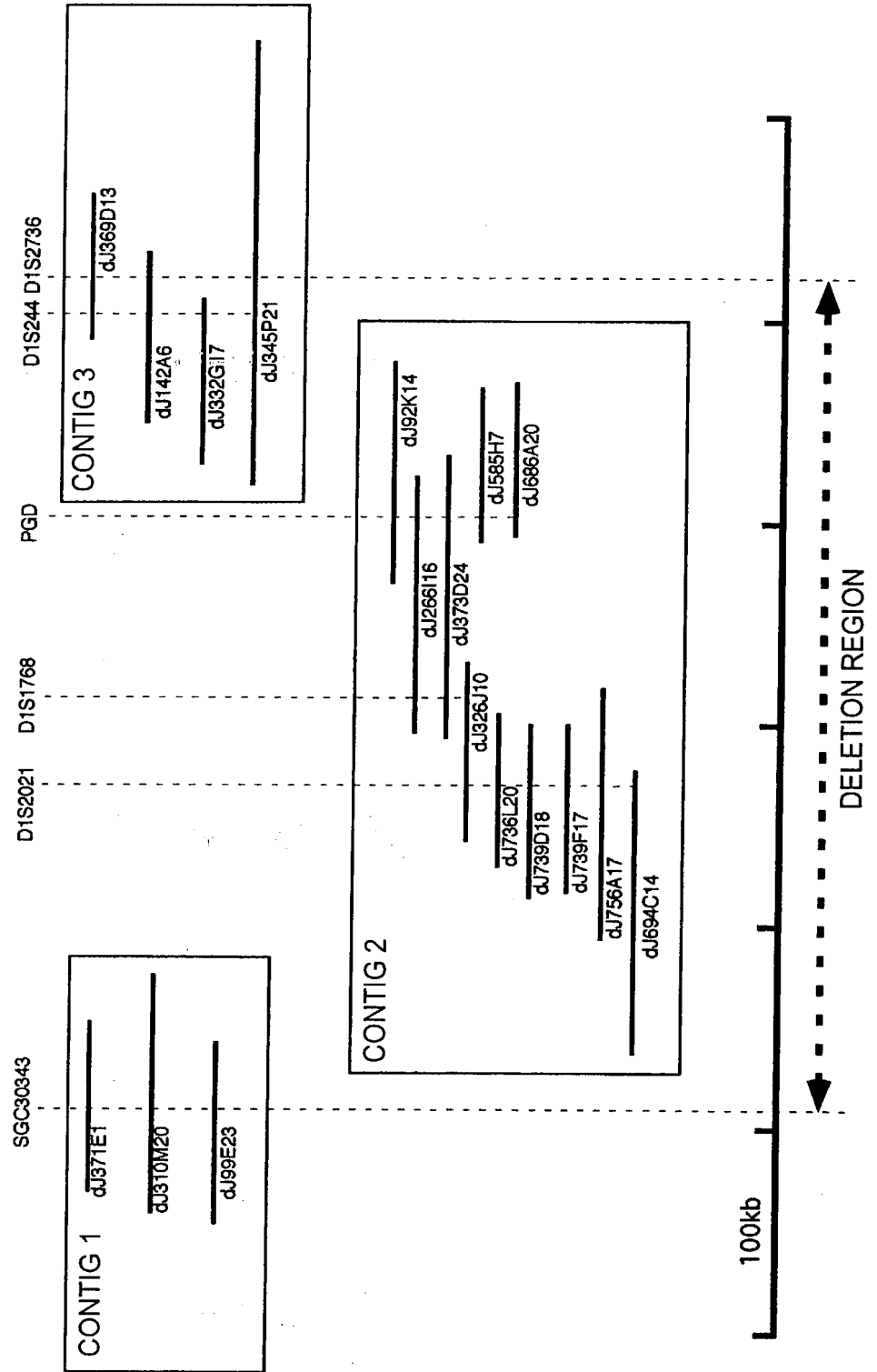


Fig. 3



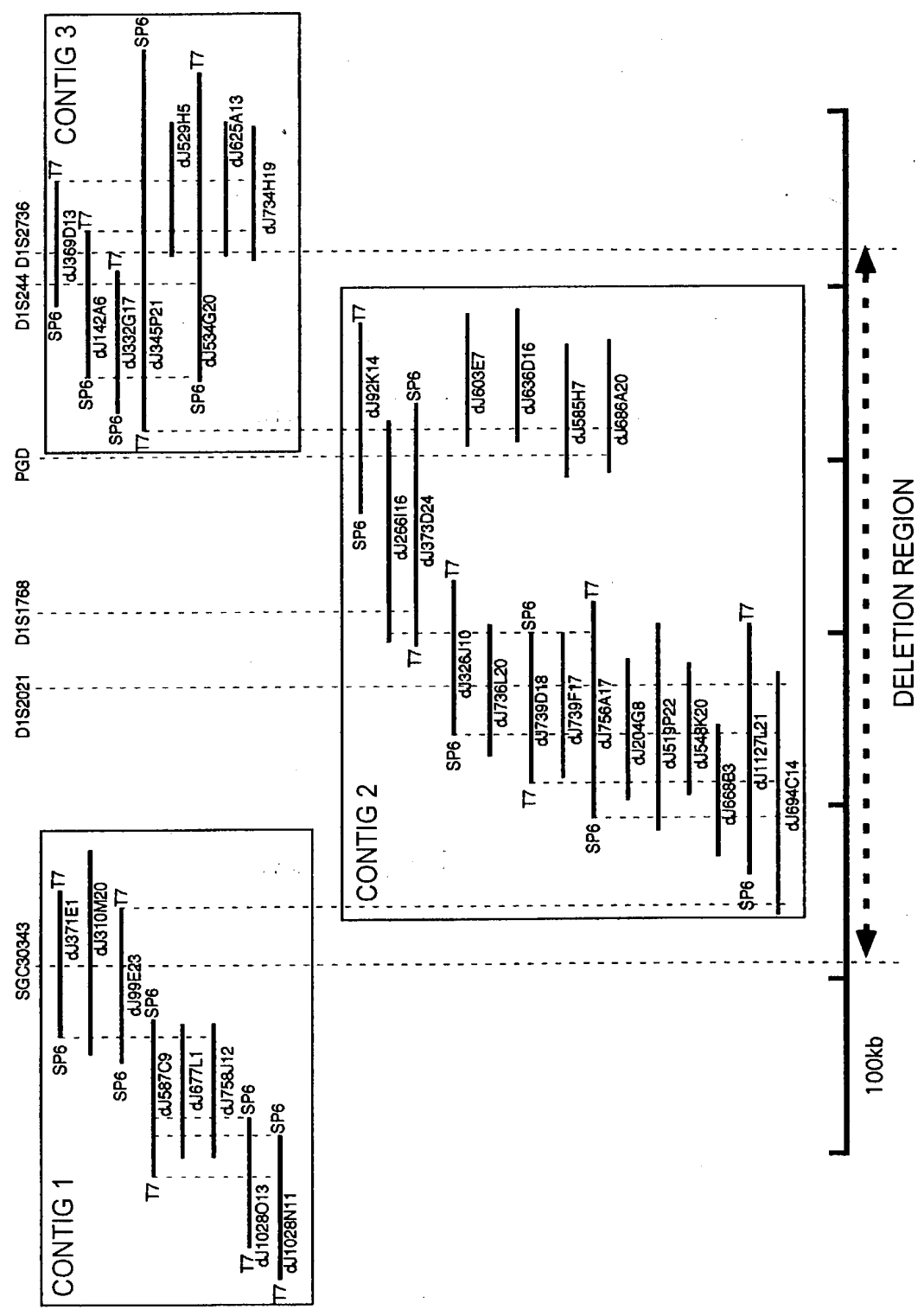
**Fig.4**

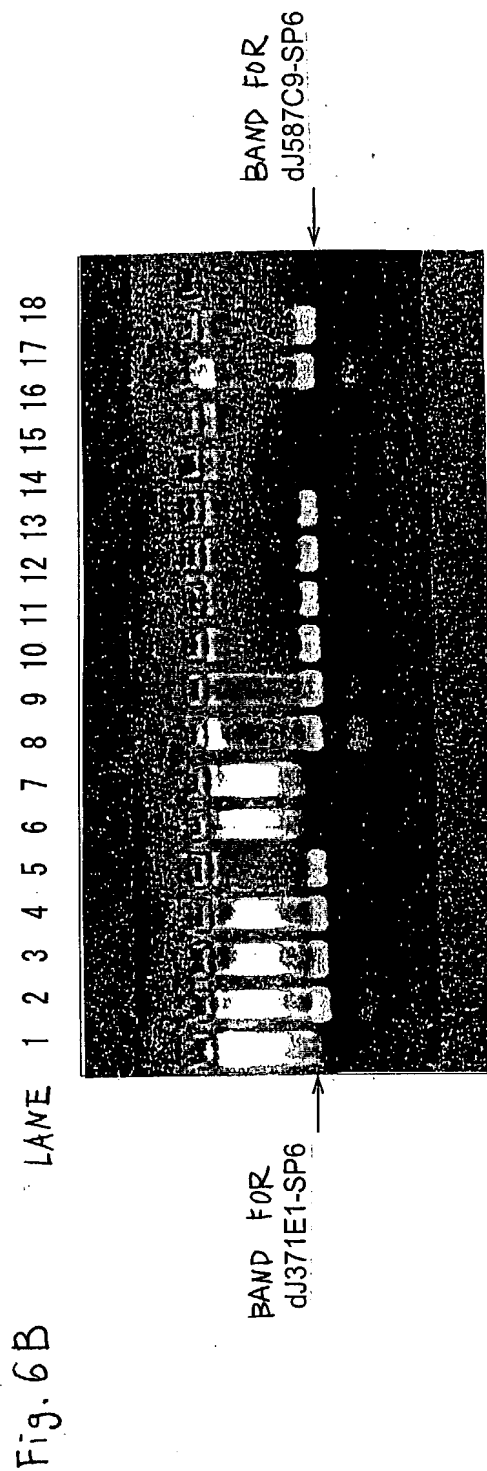
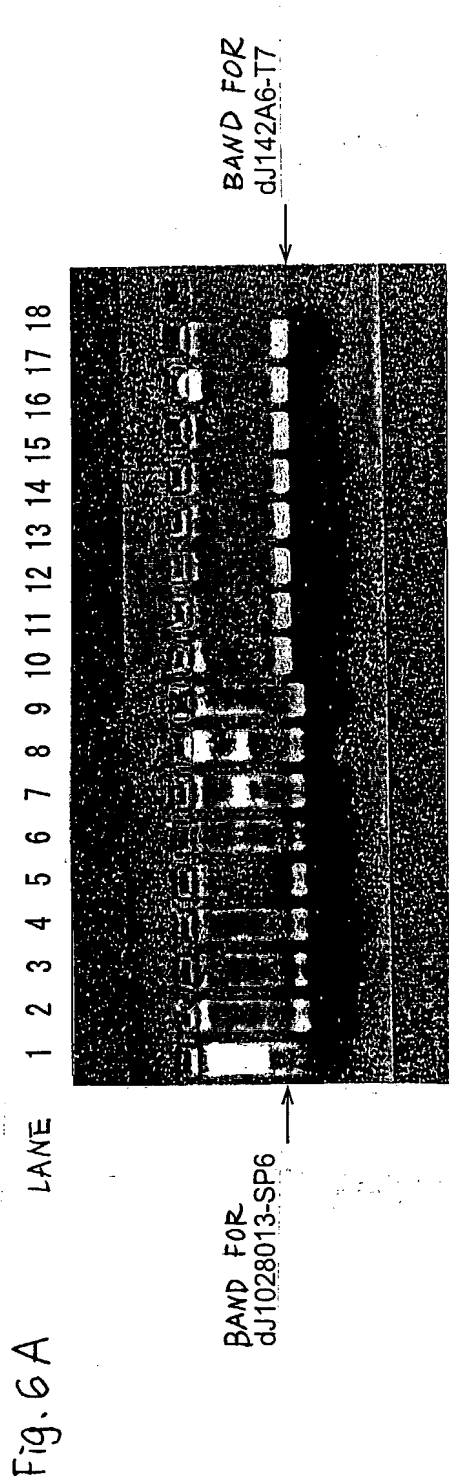
PAC CLONES OBTAINED BY 1ST SCREENING AND CONTIGS



**Fig.5**

FINALLY OBTAINED 34 PAC CLONES AND CONTIGS





Title: HUMAN 1p36 HOMOZYGOUS DELETION REGION  
Inventor: Akira NAKAGAWARA  
Atty Docket No.: 7388/72613  
Fitch, Even, Tabin & Flannery  
202-419-7000

Fig.7

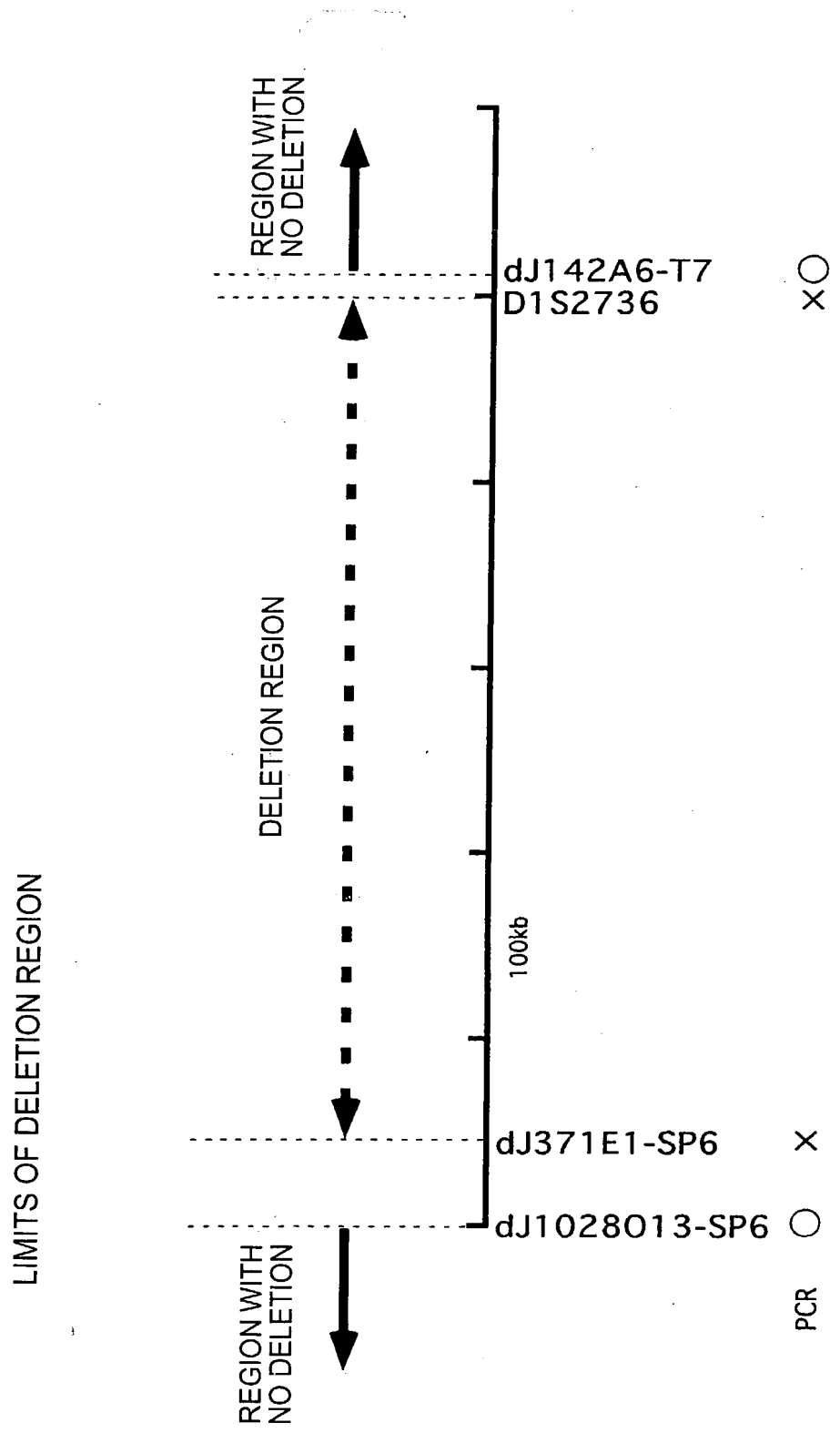




Fig. 8

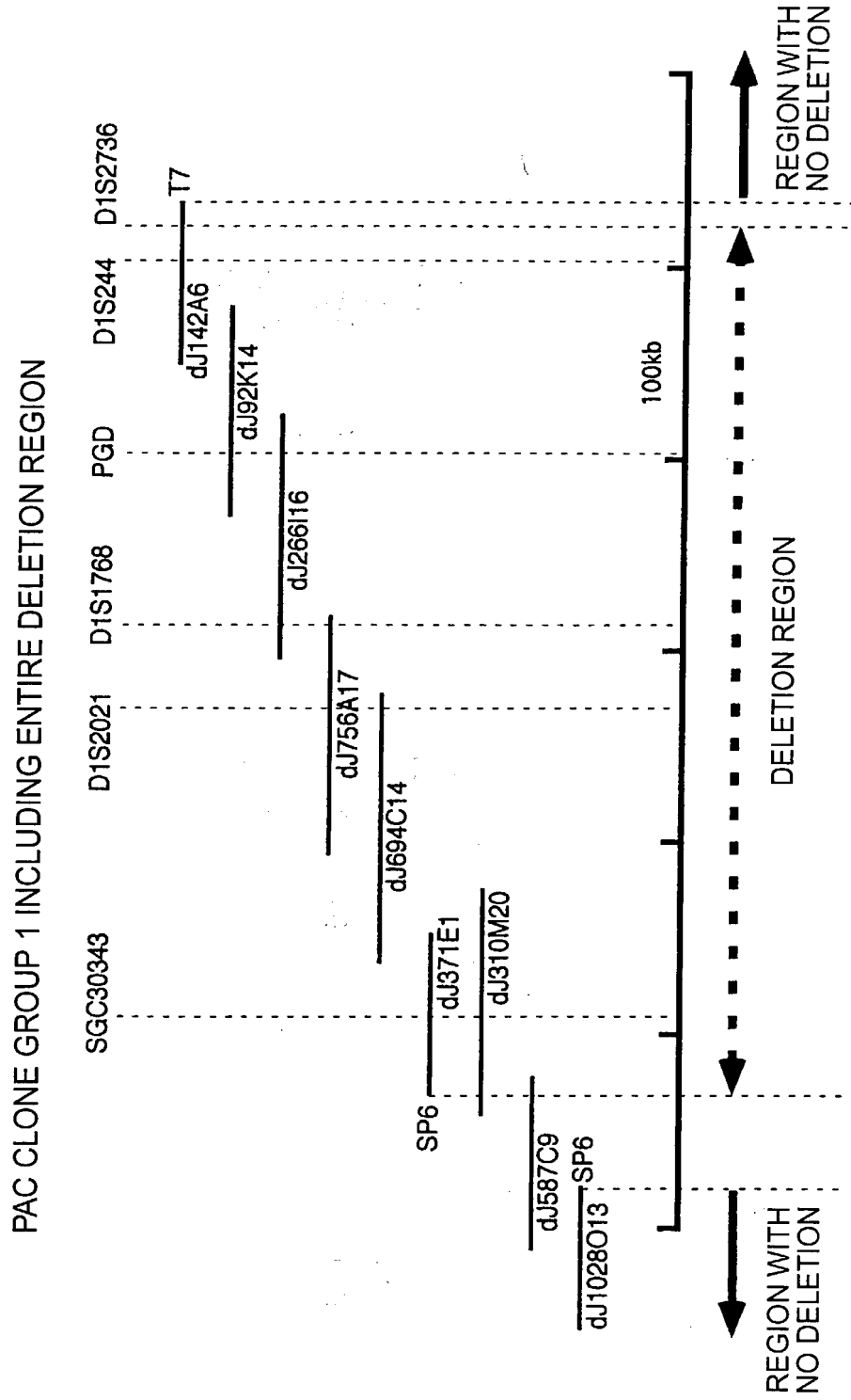
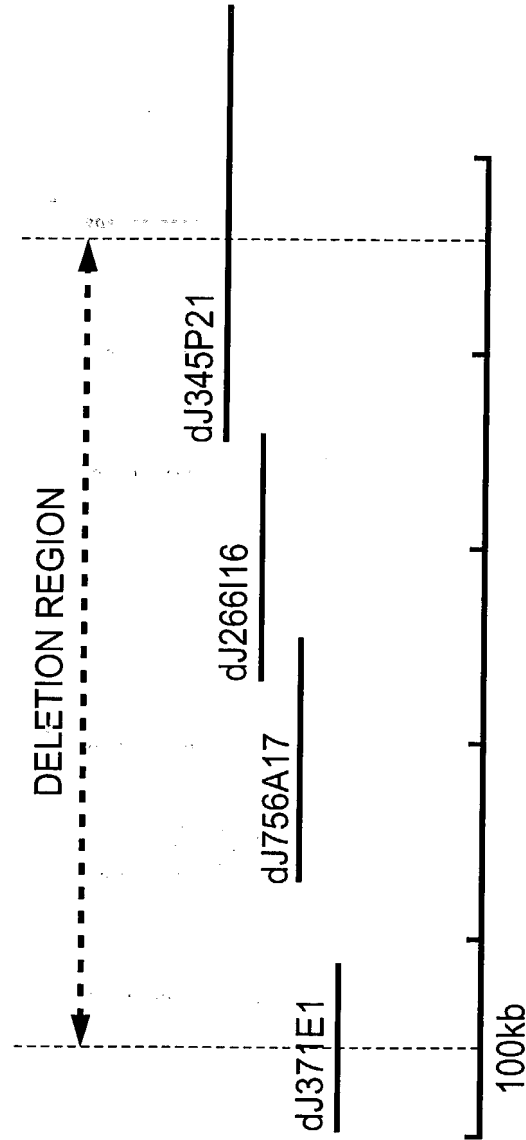


Fig. 9

PAC CLONE GROUP 2 INCLUDING ENTIRE DELETION REGION



## Combined Declaration and Power of Attorney

As a below named inventor, I hereby declare that:

This declaration is of the following type:

☐ original    ☐ supplemental

☒ national stage of PCT

☐ divisional    ☐ continuation    ☐ continuation-in-part

My residence, post office address and citizenship are as stated next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**HUMAN 1p36 HOMOZYGOUS DELETION REGION**

the specification of which

☐ is attached hereto.

☐ was filed on \_\_\_\_\_  
as United States Application Serial Number \_\_\_\_\_ and,  
was amended on \_\_\_\_\_ (if applicable).

☒ was filed on August 31, 2000  
as PCT International Application Number PCT/JP00/05930 and,  
was amended under PCT Article 19 on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority under Title 35, United States Code, Section 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

### PRIOR FOREIGN APPLICATIONS, BENEFIT CLAIMED UNDER 35 USC §119(a)

Application Number	Country	Date of Filing (Day/Month/Year)	Priority Claimed Under 35 USC 119
<u>P1999-245962</u>	<u>Japan</u>	<u>31 / August / 1999</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
<u>P2000-136266</u>	<u>Japan</u>	<u>09 / May / 2000</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

**RECEIVED**

(Application No.)

(Application No.)

(Application No.)

(Application No.)

(Filing Date)

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS  
DESIGNATING THE U.S., BENEFIT CLAIMED UNDER 35 USC §120

(Filing Date)


(Application No.)

(Status: Patented, Pending, Abandoned)

Customer Number 22242

Direct Telephone Calls to: **Kendrew H. Colton at telephone number 202-419-7000**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full Name of sole or first inventor <b>1-00 Akira NAKAGAWARA</b>	
Inventor's signature 	Date February 15, 2002
Residence Chuo-ku, Chiba-shi, <u>Chiba</u> , Japan	
Citizenship Japan <b>JPX</b>	
Post office address c/o Chiba Cancer Center Research Institute, 666-2, Nitona-cho, Chuo-ku, Chiba-shi, Chiba 260-0801 Japan	

SEQUENCE LISTING

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FP00-0197-00

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<212> DNA

<213> Homo sapiens

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<213> Homo sapience

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FP00-0197-00

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FP00-0197-00

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FP00-0197-00

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FP00-0197-00

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FP00-0197-00

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FP00-0197-00

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FP00-0197-00

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